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AWARD NUMBER DAMD17-97-1-7232

TITLE: Targeted Gene Therapy for Breast Cancer

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REPORT DATE: August 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander  
U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5400

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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE August 1998		3. REPORT TYPE AND DATES COVERED Annual (1 Aug 97 - 31 Jul 98)
4. TITLE AND SUBTITLE  Targeted Gene Therapy for Breast Cancer			5. FUNDING NUMBERS  DAMD17-97-1-7232	
6. AUTHOR(S)  Jinha M. Park				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)  University of Southern California Los Angeles, California 90031			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)  U.S. Army Medical Research And Materiel Command ATTN: MCMR-RMI-S 504 Scott Street Fort Detrick, Maryland 21702-5400			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			19990115 086	
12a. DISTRIBUTION / AVAILABILITY STATEMENT  Distribution authorized to U.S. Government agencies only (proprietary information, Aug 98). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) The amplification of the HER-2/ <i>neu</i> oncogene is a frequent genetic alteration found in 25-30% of all breast cancer cases and has been correlated with shortened disease-free and overall survival. The alteration leads to increased copy number of the gene and subsequent overexpression of the protein on the surface of cancer cells. The extracellular domain (ECD) of the protein acts as a receptor for transmission of growth regulatory signals into the cell. High numbers of HER-2/ <i>neu</i> proteins on these particular cancer cells could be used as targets for the refinement of a more specific cancer therapy. The delivery of therapeutic genes to cancer cells overexpressing breast cancer cells mediated by engineered forms of HER-2/ <i>neu</i> monoclonal antibodies is the goal of this project. We have generated four novel monoclonal antibodies (mAbs) targeted to the HER-2/ <i>neu</i> ECD. Two mAbs, 5A7 and 11F11, were produced by hybridomas derived from mice immunized by a recombinant HER-2/ <i>neu</i> ECD tagged by a polyhistidine peptide. Two other mAbs, 8H11 and 10H8, were produced by hybridomas derived from mice immunized with live HER-2/ <i>neu</i> overexpressing cell lines. All four mAbs have been extensively characterized by immunohistochemistry, immunoblotting, and antibody internalization assays. Only 8H11 and 10H8 can localize to the surface of live HER-2/ <i>neu</i> cells and subsequently be internalized by endocytosis. In order to generate a novel delivery vehicle to target cancer cells specifically, we have isolated the genes encoding the variable regions of the two best candidate mAbs, 8H11 and 10H8. By reverse-transcriptase polymerase chain reaction, we have isolated and sequenced the coding regions of both the variable heavy and variable light chains of both mAbs.				
14. SUBJECT TERMS Breast Cancer Gene Therapy HER-2/ <u>neu</u>			15. NUMBER OF PAGES 15	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Limited	

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John F. Park 8/19/98  
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## INTRODUCTION.

Amplification of the *HER-2/neu* oncogene is a genetic alteration which is correlated with poor prognosis in breast cancer. The *HER-2/neu* protein is a cell-surface receptor over-expressed in 25-30% of breast cancers. The high levels of the receptor protein increase the malignant potential of cells with *HER-2/neu* amplification, a reason attributed to the shortened overall survival of patients with this gene alteration in their tumors. The abundance of the receptor presents an attractive target for the specific delivery of novel therapeutics to the cells. I have generated four novel monoclonal antibodies, 5A7, 8H11, 11F11, and 10H8, against the extracellular domain of this receptor (ECD). The results of the project include the development of monoclonal antibodies (mAbs) to *HER-2/neu*, characterization of their binding to human carcinoma cell lines, and the development of engineered forms of the binding region of the mAbs.

All four have been screened from hybridoma supernatants against an NIH3T3 cell line (NIH/189) over-expressing the *HER-2/neu* receptor and counter-screened against a wild-type NIH3T3 cell line. Immunohistochemical analysis shows binding to *HER-2/neu* in a frozen breast cancer biopsy by all four mAbs. Only three monoclonal antibodies identify the receptor by Western Blot suggesting that abrogation of a native conformational epitope prevents binding by the 8H11 antibody. Two mAbs, 8H11 and 10H8 can bind to the membranes of live SKBR-3 human breast cancer cells by indirect immunofluorescence.

A cellular radioimmunoassay shows that two of the mAbs, 8H11 and 10H8, are bound to *HER-2/neu* over-expressing cells and internalized by receptor-mediated endocytosis. A method to detect internalization of mAbs directed against cell-surface antigens has been used previously to identify anti-*HER-2/neu* mAbs with this characteristic. mAbs 8H11 and 10H8 were labeled with <sup>125</sup>I and tracked for intracellular trafficking. Both antibodies show that by 24 hours of incubation on NIH/189 cells, more than 50% of surface mAb has been internalized by receptor-mediated endocytosis. These mAbs show promise in the specific delivery of gene therapy vectors, which are also internalized by their specific host targets by receptor-mediated endocytosis, to *HER-2/neu*-over-expressing cells.

Monoclonal antibodies with the potential to specifically target human breast cancer cells have been generated and characterized for future use as a therapeutic reagent. Two have been discovered that bind to and are internalized by *HER-2/neu*-over-expressing cells. The genes encoding the mAbs' variable fragments have been isolated and sequenced. Future directions of this project will be to express engineered mAbs and use these minimal targeting moieties to redirect the infectivity of vectors for gene therapy.

## PROGRESS DURING YEAR 01.

### Technical Objective 1-2: Production of monoclonal antibodies to ECD of p185<sup>HER-2/neu</sup>.

**Task 1:** Month 0: Bacterial expression and metal-chelate affinity chromatographic purification of ECD of p185<sup>HER-2/neu</sup> completed.

Produce monoclonal antibodies to the extracellular domain (ECD) of *HER-2/neu* (c-erb B-2). Production of monoclonal *HER-2/neu* antibodies will involve one of two strategies. Animals will be immunized with either purified, recombinant *HER-2/neu* extracellular domain (ECD) or live cells overexpressing *HER-2/neu*. In preliminary results we have used both approaches to produce the first four candidate monoclonal antibodies.

A recombinant extracellular domain (ECD) of *HER-2/neu* was cloned and expressed in a bacterial expression system. The ECD protein was purified by metal chelate affinity chromatography using its histidine tag leader peptide. The ECD protein and, in later protocols, live *HER-2/neu* overexpressing cells were used to immunize BALB/c mice.

**Task 2:** Months 1-7: Production and selection of hybridomas producing monoclonal antibodies to the ECD of p185<sup>HER-2/neu</sup>. Characterization of antibodies and epitope mapping to specific region of p185<sup>HER-2/neu</sup> protein.

Spleen cells were fused to Sp2/0-Ag14 murine myeloma cells using polyethylene glycol and hybridomas selected in HAT medium. HER-2/*neu* antibody secreting hybridomas were screened by cell ELISA and confirmed by immunohistochemistry on frozen breast cancers positive for HER-2/*neu*. Positive hybridoma cell lines were cloned twice by limiting dilution.

Isolated 5A7, 11F11, 8H11 and 10H8 monoclonal antibodies were characterized for their ability to specifically recognize HER-2/*neu* protein by Western immunoblot and immunohistochemistry and to bind to viable tumor cells overexpressing HER-2/*neu* by indirect immunofluorescence assay (Table 1). Western immunoblot showed that three of the monoclonal antibodies, 5A7, 10H8, and 11F11 bound p185 HER-2/*neu* protein on nitrocellulose blots after SDS-PAGE and transfer (Figure 1). 8H11 did not show any binding to p185<sup>HER-2/neu</sup> by Western blot but did specifically bind p185<sup>HER-2/neu</sup> in immunoprecipitation assays (data not shown).

Immunohistochemical staining using each of the isolated monoclonal mouse antibodies was performed as described in detail elsewhere (1) using frozen and paraffin-embedded, formalin-fixed, breast cancer specimens to assess the presence and distribution of HER-2/*neu* oncoprotein in the tumor cells. Breast cancers, previously determined by Western immunoblot and Northern blot analysis to have low expression of HER-2/*neu*, did not show membrane staining for HER-2/*neu*, while breast cancers, previously determined by Western immunoblot and Northern blot analysis to have overexpression of HER-2/*neu*, did show high levels of membrane staining in tumor cells but not in other cells. All four monoclonal antibodies showed similar results.

### **Technical Objective 3: Evaluation of receptor-mediated endocytosis.**

**Task:** Months 8-10: Screening of monoclonal antibodies for internalization potential.

Characterize extracellular domain antibodies demonstrating their ability to bind living breast cancer cells and to be internalized by these cells. We have used immunofluorescence with live cells to assess binding to viable tumor cells and radioimmunoassay to assess receptor-antibody cellular trafficking. Preliminary data is provided below for the first four monoclonal antibodies.

**BINDING TO VIABLE TUMOR CELLS.** In order to evaluate monoclonal antibody binding of HER-2/*neu* in viable tumor cells (no fixatives used), cultured normal HMEC cells (no overexpression) and breast cancer cells with overexpression (SK-BR-3) were treated with each monoclonal antibody and were visualized by indirect immunofluorescence. Two of the antibodies, 8H11 and 10H8, showed strong membrane staining in viable tumor cells, whereas two of the antibodies, 5A7 and 11F11, showed no immunofluorescence. Interestingly, the two antibodies showing membrane staining in viable cells had areas of apparent concentration of fluorescent signal consistent with "capping" observed during receptor-mediated endocytosis (see subsequent preliminary results for supporting data). This contrasts with the distribution of immunostaining on fixed breast tumors. Fixed breast cancers with overexpression show relatively uniform membrane staining over the surface of the cell.

**RADIOIMMUNOASSAY DETERMINATION OF RECEPTOR TRAFFICKING.** The two antibodies which bound living tumor cells were further evaluated to assess receptor-mediated (or receptor-antibody mediated) endocytosis. The rates of internalization and catabolism of these antibodies were analyzed by cellular radioimmunoassay (2). 8H11 and 10H8 were labeled with <sup>125</sup>I using the Iodo-Gen method (Pierce, Rockford, IL) and assessed for intracellular trafficking. Briefly, 100 ug of antibody were added to 0.5 mCi of Na<sup>125</sup>I in a glass tube coated with 10 ug Iodo-Gen for 10 minutes at room temperature. Free Na<sup>125</sup>I was removed by chromatography on a PD-10 column (Pharmacia), and eluted fractions were pooled and stored at 4°C.

The preliminary results (Figures 2 and 3) showed a steady decrease in surface bound radioactivity with a reciprocal increase in supernatant radioactivity for both 8H11 and 10H8. Intracellular cpm peaked at 25% (2 hours) and 15% (4 hours), respectively, for 8H11 and 10H8. After 24 hours the percentage of total radioactivity remaining cell associated (i.e., cell surface plus intracellular cpm) was 49% and 37% for 8H11 and 10H8, respectively. Conversely, after 24 hours 51% and 63% of total culture cpm were found within the supernatant for 8H11 and 10H8, respectively. Supernatants were treated with 25% TCA to determine the proportion of supernatant radioactivity contributed by intact antibody (TCA precipitable) and by small-molecular-weight metabolites (TCA soluble). TCA-soluble cpm progressively increased during the 24-hour period for both antibodies, reaching peak values of 45% and 47% of total culture cpm, respectively. Hence, approximately 45% of  $^{125}\text{I}$ -8H11 and 47% of  $^{125}\text{I}$ -10H8 were internalized, degraded, and exocytosed by the tumor cells in 24 hours.

Cellular radioimmunoassays demonstrated prompt internalization of  $^{125}\text{I}$ -labeled antibodies after binding to cells, followed by degradation and deiodination and exocytosis of the radiolabel. Presumably the internalized antibody was routed to lysosomes, as seen previously for two other antibodies (2), where the degradation occurred. This suggests that the most effective molecularly constructed vector might be one that incorporates a membrane penetrating protein as part of the construct to prevent degradation in the lysosome.

To our knowledge no other group has characterized the internalization and catabolism of any monoclonal antibody used as the basis for engineering of single-chain antibodies incorporated into gene therapy vectors. We think that this type of analysis will prove to be useful in the selection and rational design of gene therapy vectors.

#### **Technical Objective 4-5: Construction of engineered retroviral vector.**

**Task 1:** Month 11-14: Construction of single-chain monoclonal antibodies (scFv).

Isolate and characterize the genes coding for light-chain and heavy-chain variable domains of monoclonal antibodies which show desirable breast cancer binding and internalization characteristics. The cDNAs encoding the variable heavy and variable light chain domains will be cloned and characterized for each monoclonal antibody with favorable binding characteristics recognizing viable tumor cells. In order to demonstrate this approach preliminary results are provided for the two antibodies (8H11 and 10H8) recognizing HER-2/*neu* in viable tumor cells.

Two variable fragments of each 8H11 and 10H8 monoclonal antibody have been cloned from the hybridomas using the polymerase chain reaction (PCR). Total RNA was extracted by the TRIzol method (GIBCO). The RNA was converted to cDNA by Moloney murine leukemia virus reverse transcriptase. Degenerate oligonucleotides coding for the N-terminus of the variable heavy ( $V_H$ ) and variable light ( $V_L$ ) chain genes and specific oligonucleotide primers to the constant regions of the respective variable chains were used to amplify 400 bp fragments from the cDNA (Table 2) Appendix B) (3). Sequencing of each PCR reaction of each variable heavy and light chains were consistent with immunoglobulin consensus sequences as compiled in the Kabat Database (4).

## **CONCLUSIONS.**

Our preliminary work demonstrates 1.) our ability to generate monoclonal antibodies of the desired specificity, 2.) the ability of two of our four antibodies to bind living cells (as opposed to fixed cells), 3.) the favorable internalization features of two of our antibodies after binding to viable cells, and 4.) isolation and characterization of cDNAs coding for the heavy-chain and light-chain domains from both of the antibodies with favorable internalization characteristics.



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3. S. Dubel, et al., *J. Immunol. Methods* **175**, 89 (1994).
4. E. A. Kabat, T. T. Wu, H. M. Perry, K. S. Gottesman, C. Foeller, *Sequences of Proteins of Immunological Interest*. E. A. Kabat, Ed. (US Department of Health and Human Services, US Government Printing Office, ed. 5th, 1991).

## APPENDICES.

1. Table 1. Characteristics of HER-2/*neu* Monoclonal Antibodies.
2. Figure 1. Western blot analysis of mAb binding to total protein lysates.
3. Figure 2 A & B. Internalization and Catabolism of mAb 8H11 by NIH/189 Cells.
4. Figure 3 A & B. Internalization and Catabolism of mAb 10H8 by NIH/189 Cells.
5. Table 2. Primers used to isolate variable heavy and light chains of mAb 8H11 and mAb 10H8.

**Table 1. Characteristics of HER-2/*neu*  
Monoclonal Antibodies**

<b>Characteristic</b>	<b>5A7</b>	<b>11F11</b>	<b>8H11</b>	<b>10H8</b>
Immunogen	* ECD protein	* ECD protein	Live cells	Live cells
Isotype and subclass	IgG1/κ	IgG1/κ	IgG1/κ	IgG2a/κ
Protein ELISA	+	+	-	+
NIH 3T3 wt ELISA	-	-	-	-
NIH/189 ELISA	+	+	+	+
IHC on frozen tissue	+	+	+	+
IHC on paraffins	+	+	+/-	+
Western blots	+	+	-	+
Immunoprecipitation	-	-	+	+
Indirect immunofluorescence	-	-	+	+
Endocytosis	-	ND	+	+

\* ECD = Extracellular domain  
ND = Not determined

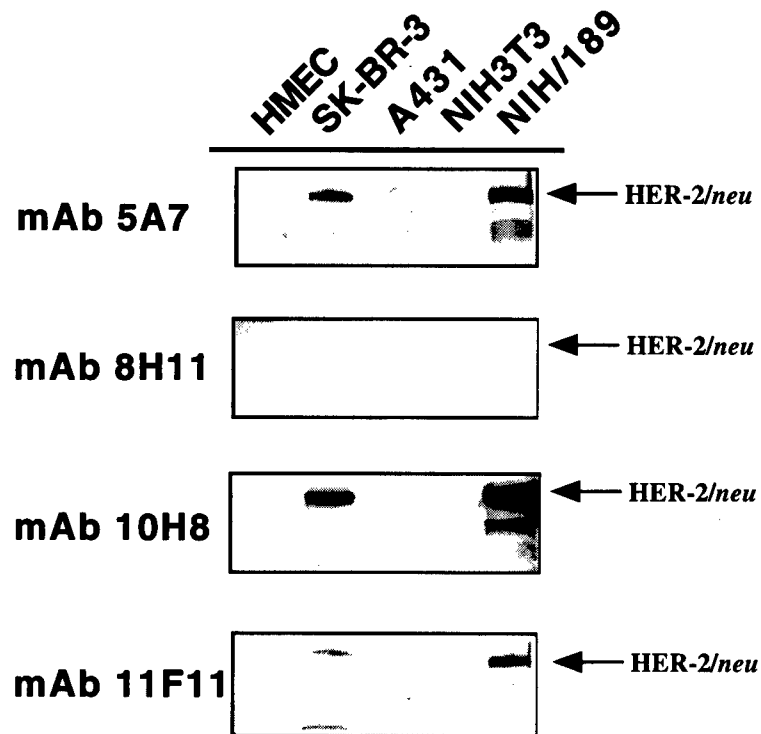
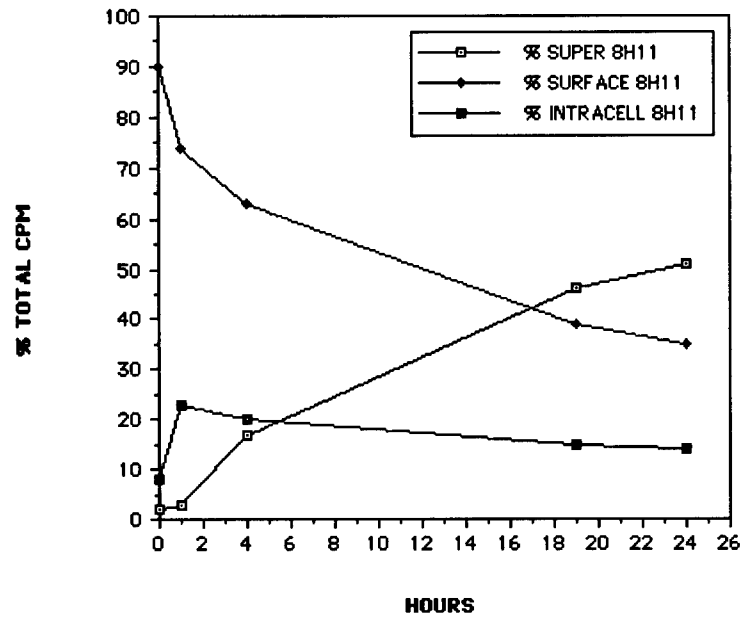


Figure 1. Western Blot analysis of mAb binding to total protein lysates. HMEC, human mammary epithelial cells; SK-BR-3, high HER-2/*neu*-expressor breast cancer cells; A431, human epidermoid cancer cells; NIH3T3, mouse embryo fibroblasts; NIH/189, NIH3T3 cells over-expressing HER-2/*neu*.

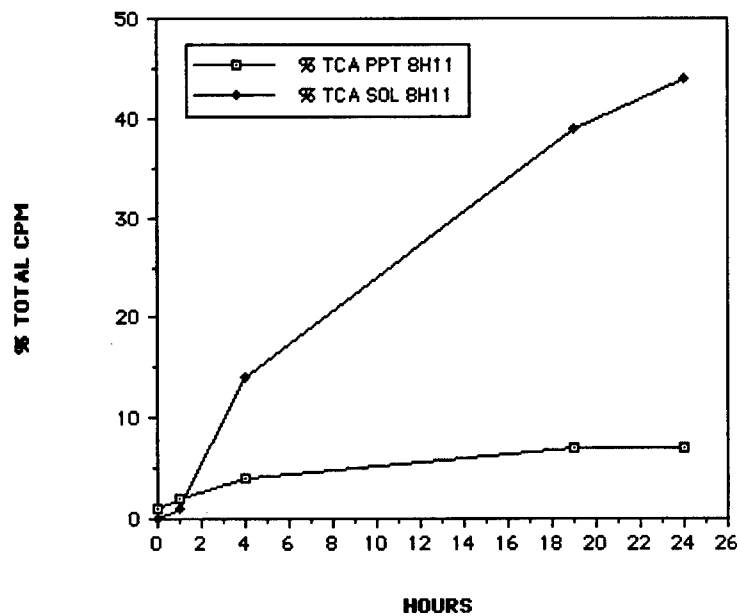
**Figure 2.** Internalization and Catabolism of mAb 8H11 by NIH/189 cells. *Top Panel (A).* mAb 8H11 labeled with  $^{125}\text{I}$  was incubated with NIH/189 cells overexpressing HER-2/*neu* and fractions followed for evidence of receptor-mediated endocytosis. Mean cpm for triplicate wells were counted and graphed. Surface bound mAb decreases over 24 hours while supernatant cpm increases. *Bottom Panel (B).* Supernatant cpm was separated by 25% TCA precipitable cpm (intact mAb) and TCA soluble cpm (small MW metabolites of mAb). Increased TCA soluble cpm shows degradation and exocytosis of internalized mAb 8H11.

**Figure 2. Internalization and Catabolism of mAb 8H11 by NIH/189 Cells**

**A.**



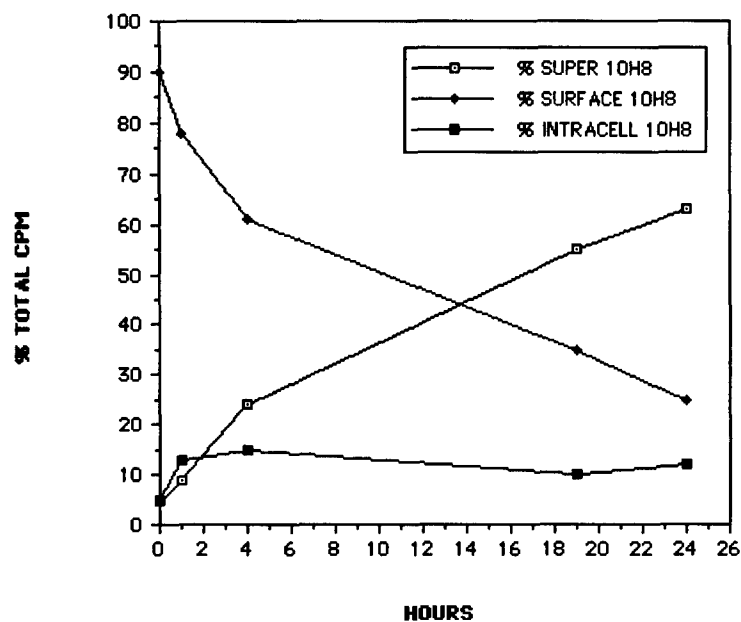
**B.**



**Figure 3.** Internalization and Catabolism of mAb 10H8 by NIH/189 cells. ***Top Panel (A).*** mAb 10H8 labeled with  $^{125}\text{I}$  was incubated with NIH/189 cells overexpressing HER-2/*neu* and fractions followed for evidence of receptor-mediated endocytosis. Mean cpm for triplicate wells were counted and graphed. Surface bound mAb decreases over 24 hours while supernatant cpm increases. ***Bottom Panel (B).*** Supernatant cpm was separated by 25% TCA precipitable cpm (intact mAb) and TCA soluble cpm (small MW metabolites of mAb). Increased TCA soluble cpm shows degradation and exocytosis of internalized mAb 10H8.

**Figure 3. Internalization and Catabolism of mAb 10H8 by NIH/189 Cells**

**A.**



**B.**

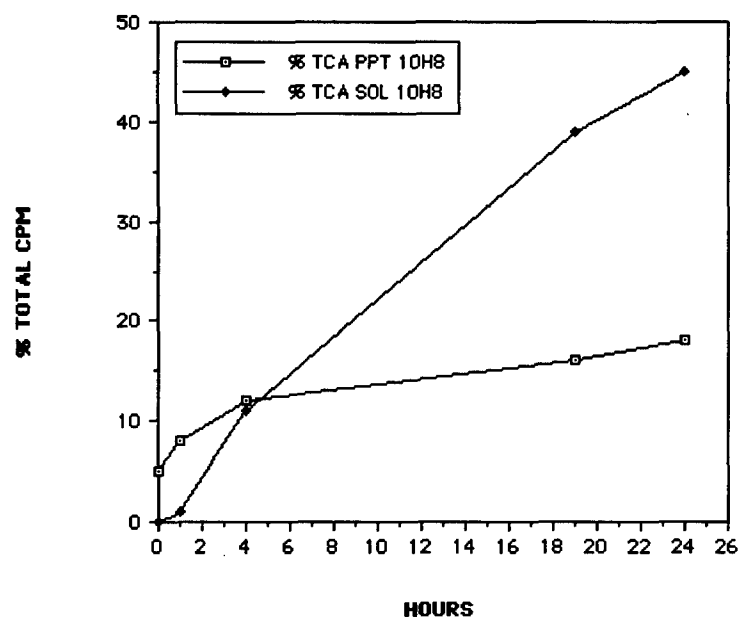


Table 2. Primers used to isolate variable heavy and variable light chains of mAb 8H11 and mAb 10H8

Primer	Description	Oligonucleotide Sequence
VHBI3	Heavy chain variable domain	5'-GAGGTGAAGCTGAGGAGTCAGGACCTAGCCTGGTG-3' (PstI site)
VHBI3c	Heavy chain variable domain	5'-AGGT(C/G)(A/C)AGCTGCAG(C/G)AGTC(A/T)GG-3' (PstI site)
VHBI3d*	Heavy chain variable domain	5'-AGGT(C/G)CAGCTGCAG(C/G)AGTC(A/T)GG-3' (PvuII site)
HCRgi2*	Gamma chain constant domain	5'-CGGAATTCAGGGGCCAGTGGATAGAC-3' (EcoRI site)
VkBI6	Light chain variable domain	5'-GGTGATATCGTGAT(A/G)AT(C/A)CA(G/A)GATGAACCTCTC-3' (EcoRV site)
VkBI7*	Light chain variable domain	5'-GGTGATATC(A/T)TG(A/C)TGACCCAA(A/T)CTCCACTCTC-3' (EcoRV site)
VkBI8	Light chain variable domain	5'-GGTGATATCGT(G/T)CTCAC(C/T)CA(A/G)TCTCCAGCAAT-3' (EcoRV site)
LCRgi2*	Kappa chain constant domain	5'-CGGAATTCGGATGGTGGGAAGATGGA-3' (EcoRI site)

\* Actual primers used in cloning VH8H11, VH10H8, VL8H11, and VL10H8.